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Antimicrobial activity of lupulone against *Clostridium perfringens* in the chicken intestinal tract jejunum and caecum

G. R. Siragusa[1]*, G. J. Haas[2], P. D. Matthews[3], R. J. Smith[3], R. J. Buhr[1], N. M. Dale[4] and M. G. Wise[5]

[1]Agricultural Research Service, United States Department of Agriculture, Poultry Microbiological Safety Research Unit, Russell Research Center, Athens, GA, USA; [2]School of Natural Sciences, Fairleigh Dickinson University, Teaneck, NJ, USA; [3]S.S. Steiner, Inc., New York, NY, USA; [4]Department of Poultry Science, University of Georgia, Athens, GA, USA; [5]bioMérieux, Bacterial Barcodes, Inc., Athens, GA, USA

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***Objectives*: Owing to the spread of antibiotic resistance among human infectious agents, there is a need to research antibiotic alternatives for use in animal agricultural systems. Antibiotic-free broiler chicken production systems are known to suffer from frequent outbreaks of necrotic enteritis due in part to pathogenic type A *Clostridium perfringens*. Hop (*Humulus lupulus*) bitter acids are known to possess potent antimicrobial activity. Lupulone was evaluated for *in vivo* antimicrobial activity to inhibit *C. perfringens* in a chick gastrointestinal colonization model.**

***Methods*: Using a week-2 per os inoculated *C. perfringens* chicken colonization model, *C. perfringens* counts in mid-intestinal and caecal contents were compared between chickens administered lupulone at 62.5, 125 and 250 ppm in drinking water versus 0 ppm control.**

***Results*: At day 22, post-hatch intestinal *C. perfringens* counts of lupulone-treated chickens were significantly lower ($P < 0.05$) than water-treated control groups in both jejunal and caecal sampling sites across all lupulone dosages tested.**

***Conclusions*: Lupulone administered through water inhibits gastrointestinal levels of inoculated pathogenic clostridia within the chicken gastrointestinal tract. Lupulone was effective within the chemically complex mixture of material within the gastrointestinal tract, thereby making this agent a target of further research as an antibiotic alternative for this and possibly other intestinal infections.**

Keywords: *Humulus lupulus*, hops, β-acids, plant-derived antimicrobials, antibiotic alternatives, poultry necrotic enteritis, *Clostridium* spp.

Introduction

Bitter acid constituents of the hop plant (*Humulus lupulus*) possess potent antimicrobial activity against a range of microorganisms, yet do not inhibit yeasts.[1–6] Antimicrobial applications of hop resins include antiprotozoal, anticlostridial and antiviral activities, various food applications, use as poultry and animal feed additives, and use as a source of potential new antibiotics.[7–9]

Bacterial species with reported susceptibility to hop bitter acids include *Lactobacillus* spp., *Pediococcus* spp., *Bacillus* spp., *Clostridium botulinum*, *Clostridium difficile*, *Mycobacterium tuberculosis*, antibiotic-resistant forms of *Enterococcus* spp., *Staphylococcus aureus* and *Helicobacter pylori*.[2]

Hop bitter acids are broadly classed into two groups: α-acids and β-acids. α-Acids (humulones) and their water soluble isomers and the iso-α-acids (isohumulones) are the major bitter flavour components in beer brewing. Hop bitter acids, from 1 to 250 ppm, act as ionophores against the Gram-positive bacterial cell wall by de-energizing the transmembrane potential causing ATP leakage and cell death.[10,11] The antimicrobial activity of β-acids is attributed to a series of congeners that share a basic alicyclic structure of 2,4-cyclohexadiene-1-one (Figure 1).

*Correspondence address. Agtech Products, Inc., W227 N752 Westmound Dr, Waukesha, WI 53186, USA. Tel: +1-262-521-1717; Fax: +1-262-521-2442; E-mail: gsiragusa@agtechproducts.com

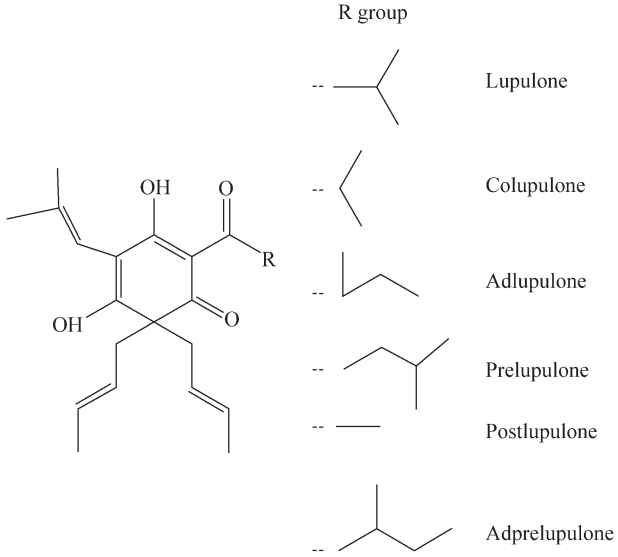

Figure 1. Structure of major components of the lupulone fraction of the hop plant (*Humulus lupulus*).

β-Acids (lupulone) and its congeners (adlupulone, colupulone and so on; see Figure 1) contribute less bitter flavour than the α- and iso-α-acids, but possess greater antimicrobial activity on a per mole basis.

The practice of feeding non-therapeutic antibiotic growth promoters (AGPs) to livestock and poultry has come under scrutiny as the ecological catalyst or selective pressure for the selection of antimicrobial-resistant microorganisms' resistance genes that could impact human antibiotic therapy. Consequently, the use of AGPs in animal feeds is being reduced, or, in some nations, banned outright.[12] One consequence of AGP withdrawal from intensive poultry rearing (i.e. drug-free rearing) is the increased intestinal carriage level of *Clostridium perfringens*, resulting in sporadic outbreaks of necrotic enteritis.[13] Subsequent onset of this potentially fatal disease forces the use of conventional therapeutic antibiotics on a whole-flock scale, thereby defeating the purpose of drug-free rearing.

In response, scientific communities are actively searching for alternatives to AGPs for use in food animal production and preharvest food safety. Because lupulone is a potent anticlostridial agent *in vitro*, we hypothesized that lupulone (β-acids) of hop extract would inhibit or reduce levels of intestinal *C. perfringens* in a broiler chicken challenge model.

Materials and methods

Microorganisms

Challenge strains were type A *C. perfringens* isolated from commercially reared broiler chickens diagnosed with necrotic enteritis grown under drug-free all-vegetable diet conditions previously described.[14] All bacteria were part of the ARS-USDA culture collection of the Poultry Microbiological Safety Research Unit (Athens, GA, USA). Strains were maintained in cooked meat medium and propagated in pre-reduced brain heart infusion broth under anaerobic conditions for 16 h at 37°C prior to challenge dosage preparation.

Challenge inocula preparation

Three challenge strains of *C. perfringens* (lab strains 726, 509 and 1113) were propagated under anaerobic conditions in pre-reduced cooked meat medium (Oxoid, Inc., Basingstoke, UK) for 16 h at 37°C in screw cap tubes. Cells were harvested by centrifugation (4500 **g** at 4°C) and decanted by gently resuspending the pellet to approximately 1/10 original volume in physiological saline to a density range of $\log_{10}$ 8–9 cfu/mL.

Chicken challenge model

All animal experiments were conducted in compliance with Agricultural Research Service (ARS)-USDA Laboratory Animal Care and Use Committee standards for care, feeding, euthanasia and disposal. Our challenge model, similar to that described by La Ragione *et al.*,[15] used no *Eimeria* spp. co-infection. Day-of-hatch broiler chickens (*Gallus gallus domesticus*) were obtained from a local commercial hatchery and transported to the ARS-Poultry Microbiological Safety Research Unit experimental farm. Chicks were reared under 24 h of low-level lighting in environmentally controlled 49 ft^2 floor pens (starting at 35°C and lowered to 32°C at 2 weeks of age) on wood shavings, watered and fed non-medicated corn-soy-based diets *ad libitum* for 13 days. Non-medicated broiler chicken starter diet feed (University of Georgia, Department of Poultry Science, Athens, GA, USA; corn-soy based, 23% protein, 6% fat, 2.5% fibre, 1.0% calcium, 0.48% available phosphorous; 3100 kcal/kg) was fed throughout the experiment. After 13 days, chickens were randomly placed in groups of 10 per cage in battery cages and housed in environmentally controlled rooms under 24 h continuous low-level lighting and were provided with standard grow out non-medicated feed and watered *ad libitum*. Within each of the separate cages, a four-nipple drinker line (Ziggity Systems, Inc., IN, USA) was individually connected to a carboy reservoir. The eight-cage battery unit was maintained at 32°C. On each of three successive mornings (day 14, 15, 16), all chicks were challenged with 0.1 mL of the three strain *C. perfringens* cocktail prepared by mixing equal volumes of washed cell suspensions into PBS (pH 7.2) resulting in a per bird dosage of approximately $\log_{10}$ 7 per bird total. The challenge was administered per os with a round-tipped 20 g, 1.5 inch animal feeding needle (Popper and Sons, New Hyde Park, NY, USA). Dosages were verified by plating the three strain cocktail on 5% sheep's blood agar (Remel, Inc., Lenexa, KS, USA) incubated at 37°C under anaerobic conditions for 16–20 h. On days 17 and 22, five chickens per treatment group were euthanized for testing. Chickens were stocked at 10 birds per treatment cage in each replication. Experiments were replicated twice.

Lupulone preparation

β-Hop extract was obtained as a 30% potassium salt of lupulone in food grade propylene glycol (S.S. Steiner, Inc. Yakima, WA, USA) prepared using supercritical CO_2 extraction of hops. Hop extracts are listed as 'generally recognized as safe' substances for food use by the United States Food and Drug Administration. HPLC analysis of the hops preparation confirmed the following composition: 29.9% lupulone, (HPLC, w/w), 0.2% iso-α-acids, 1.2% deoxy-α-acids components. The lupulone preparation was diluted in tap water to obtain mixtures of 62.5, 125 and 250 ppm (154, 307 and 614 μM, respectively). Previous experimentation with broiler chickens in our labs indicated that higher concentrations were apparently unacceptable to the test fowl (data not shown). Starting at day 13, lupulone was administered to the chickens by way of the individual cage waterers as described earlier. Any remaining unconsumed

liquid was discarded daily and replaced with freshly prepared lupulone solutions. Approximate liquid consumption within each level administered was monitored in a preliminary trial.

Microbiological analysis

Birds were euthanized by cervical dislocation as per ARS-USDA standard procedures. Five birds per treated group were euthanized, sampled and analysed for clostridial counts on days 17 and 22. Within <1 h post-mortem, intestinal tract jejunum region samples and caecal samples were obtained and sealed in a plastic sampling bag. Materials were immediately transported to the lab where they were analysed for *C. perfringens* as per the method of La Ragione *et al.*[15] Approximately 1 g of sample was weighed into a screw-capped 15 mL conical centrifuge tube and diluted in PBS at a ratio of 1:10. Care was exercised to minimize headspace by using volumes of the order of 13–14 mL total. The material harvested from the jejunum (4 inches on each side of the Meckel's diverticulum) was obtained by scraping and included the lumen contents and the mucosa. Similarly, caecal contents were obtained by dissecting one or both caecal lobes and scraping material directly into a tube for dilution. Samples were homogenized by brief vortexing. Further dilution was made into PBS and spread plated to 5% sheep's blood agar, followed by 16–24 h incubation under anaerobic conditions (Anaerogen, Oxoid, Inc.). Colonies characteristic of *C. perfringens* on blood agar (double zone of haemolysis) were counted to calculate the $\log_{10}$ cfu/g of the material. Presumptive *C. perfringens* colonies were subcultured and verified as *C. perfringens* by a 16S rRNA-DNA PCR assay.[14] At the time of direct plating, a 1.0 mL subsample of the 1:10 diluted sample was inoculated into iron milk medium (11 mL per 16×125 mm screw-capped tube) and incubated at 37°C anaerobically for 16 h. In the event of direct enumeration resulting in no presumptive colonies, the incubated enrichment culture was streaked on 5% sheep's blood agar for anaerobic culture and analysis for *C. perfringens*.

Chemical analysis

Samples of β-acid solutions were held in sealed plastic jars with a minimum of headspace at 5°C for analysis by HPLC (European Brewing Convention, Analytica-EBC, 7.9 iso-α-acids and reduced iso-α-acids in Hop Products by HPLC, Nürnberg, Germany, 2004). An international calibration extract ICE-2 (obtained from The American Society of Brewing Chemists, St. Paul, MN, USA) with a specified concentration of lupulone was used as an HPLC standard of analysis.

Statistical analysis

All microbiological counts were transformed to the $\log_{10}$ cfu/g of sample. Samples that were negative by direct plating and positive upon enrichment were assigned a count of 10 (the minimum detection threshold for a 1:10 diluted sample). All samples negative by both direct and enrichment culture were assigned a value of 1 cfu/mL for the purpose of log-transformed analyses. Statistics and mean separation were performed using the non-parametric analytical tools of InStat Version 3.0 statistical software (GraphPad, Software, Inc., San Diego, CA, USA).

Table 1. Chicken challenge model average observational results

	Hop β-acid (ppm) tap-drinking water preparations			
Parameter	0	62.5	125	250
Solution pH	6.07	6.50	6.78	7.35
Final carcass weight (kg)	1.01	0.91	1.03	1.08
Per bird total liquid consumption (L)	1.61	1.61	1.48	1.09
Residual β-acids (ppm)[a] in distilled water	nt[b]	40.6	78.8	190.7
Residual β-acids (ppm)[a] trial 1	nt	10.1	27.4	29.4
Residual β-acids (ppm)[c] trial 2	nt	4.9	10.6	13.9

[a]HPLC analysis, 22 days post-preparation.
[b]Not tested.
[c]HPLC analysis 6 days post-preparation.

Results

General experimental observations are listed in the mean summary form in Table 1. It was consistently observed that test birds administered lupulone at the 250 ppm concentration consumed less liquid than any other group. In addition, it was observed that at the 250 ppm level of lupulone watering, the nipple drinkers were prone to sticking from gum formation due to low hops solubility in water. Although this did not prevent active drinking by the chickens, these line drinking nipples were changed as needed. Although less of the 250 ppm lupulone-containing water was consumed, the final bird weights were not impacted by the level of lupulone (Table 1). As the birds are not synchronized with respect to feed intake and tract emptying, we observed obvious disparities in the degree of intestinal fullness, jejunum/caecum volume, and consistency of the intestinal and caecal material itself. Within all treatment groups, all birds appeared healthy, and neither pathologies nor lesions typical of necrotic enteritis were observed within any of the dissected samples. This observation was congruent with the challenge model used not being a necrotic enteritis pathogenesis model. The pH of lupulone solutions was within the range of growth for *C. perfringens*. Lupulone levels were determined on samples day 1 lupulone preparations are reported (Table 1). The oxidative stability of lupulone in the tap water used in these experiments was less than optimal as evidenced by the detection of hulupone, lupulone oxidation products (Table 1). Given that mineral-bearing tap water was used in these experiments, the oxidation impact on the initial β-acid concentration was not unexpected. The stability of lupulone in mineral-free distilled water was confirmed through analysis (Table 1).

Lupulone was continually administered through the water supply from day 13 to day 22. All treatment groups contained some samples that required cultural enrichment in order to detect *C. perfringens* (Table 2). With a single exception from the day 17 jejunum sample within the 62.5 ppm β-acid-treated chickens, the remaining day 17 jejunum and caecal *C. perfringens* levels were lower than controls both numerically and in frequency of occurrence (Table 2). Statistical analysis of the day 17-challenged data indicated no significant ($P < 0.50$)

Table 2. Jejunal and caecal content populations of *C. perfringens* in challenged broiler chickens administered *Humulus* β-acids through drinking water

Day	Site	Lupulone (ppm)	Log_{10} cfu *C. perfringens*/g				Incidence (pos/total)
			mean	SD	median	range	
17	jejunum	0	3.33	2.28	3.62	0.00–6.83	8/10
		62.5	3.49	2.52	2.57	0.00–9.00	8/10
		125	2.62	2.38	2.54	0.00–6.85	7/10
		250	1.68	1.63	2.15	0.00–4.67	6/10
17	caecum	0	4.42	2.84	6.04	0.00–7.09	8/10
		62.5	3.79	2.78	3.92	0.00–9.00	8/10
		125	3.16	2.09	3.56	0.00–6.20	8/10
		250	2.08	2.32	1.50	0.00–6.04	5/10
22	jejunum	0	5.95	2.22	6.43	0.00–9.00	10/10
		62.5	1.42	1.25	2.15	0.00–3.00	6/10
		125	0.91	1.18	0.00	0.00–2.48	4/10
		250	1.86	1.14	2.00	0.00–3.36	7/9
22	caecum	0	6.45	1.85	6.60	0.00–9.00	10/10
		62.5	3.23	1.02	2.85	0.00–5.60	10/10
		125	1.57	1.39	2.30	0.00–3.20	6/10
		250	3.01	0.68	3.00	0.00–3.68	9/9

Data are the log_{10}-transformed counts normalized on a per gram of caecal or jejunum content obtained from replicate experiments ($n = 10$); for analytical details, refer to the Materials and methods section.

differences in the *C. perfringens* levels either in the jejunum or in the caecal sites across all levels of lupulone administration. Within day 17, jejunum and caecal samples were found in challenged birds from which no *C. perfringens* was detected. The frequency of non-detectable samples among the day 17 samples was highest in the 250 ppm β-acid-water sampled set.

Clostridium counts from treated groups sampled on day 22 all demonstrated significantly lower *C. perfringens* levels ($P < 0.01$) when compared with the 0 ppm β-acid-treated chickens for both jejunum and caecal samples (Table 2). No statistically significant differences were observed between clostridial counts from samples derived from different levels of β-acid watering: 62.5, 125, 250 ppm. The average *C. perfringens* counts from jejunum and caecal samples of the untreated bird group (log_{10} 6.20 cfu/g) were significantly greater than the (log_{10} 2.00 cfu/g) average count of the overall β-acid hop extract-treated groups.

Discussion

Frequently, antimicrobial compounds studied *in vitro* subsequently fail to demonstrate the antimicrobial activity *in vivo*. Although there may be several reasons for this phenomenon (e.g. non-specific interactions with feed or intestinal components, toxicity, short serum half-life, blood protein binding and chemical instability), here we have presented evidence that anticlostridial activity of lupulone, administered through the drinking water, is retained and functions in the chicken intestinal tract.

No statistically significant antimicrobial effect against *C. perfringens* was observed at day 17 sampling. As presumably water intake was consistent throughout the experiment, it is not apparent why this would be the case unless the levels of lupulone sufficient for an antimicrobial effect were only reached by day 22. Another factor that might have impacted antimicrobial activity is pH of the specific gut region. Hop antimicrobial activity is favoured by an acidic pH.

MICs observed were similar to previously reported antimicrobial activity of lupulone and hop extract (including lupulone) against *Clostridium* spp. *in vitro* ranging from 1 to 100 ppm. Earlier experimentation in our laboratory found that the selected challenge strains of *C. perfringens*, derived from drug-free poultry operations undergoing necrotic enteritis outbreaks, were susceptible to 8–16 μg/mL β-acid hop extract, as determined by a broth tube dilution assay.[16]

On the basis of *in vitro* experimentation, workers have reported that physiological conditions such as pH, sodium chloride and serum should not preclude lupulone antimicrobial activity. Simpson and Smith[17] determined that lipids antagonized trans-isohumulone, whereas monovalent and divalent cations had varied effects on activity. Larson *et al.*[18] demonstrated hop extracts to have greater antilisterial activity in lower pH and lower fat content foods in a study of its application to milk, cottage cheese and coleslaw. These same workers demonstrated that over an *in vitro* pH range of 4.0–7.0, activity decreased as pH increased. The pH of the chicken intestinal tract contents increases towards the caecal site from 4.5 in the crop, to 5.7–6.0 in the duodenum/jejunum, to 6.3–6.4 in the ileum/rectum and finally up to pH 7.0 or higher in the caecum.[19] Hop component interactions with differing gut pH and feed/ingesta are factors, which might be studied in future experiments.

In the current challenge model, timing of lupulone administration was planned to coincide with the 18–21 day of life onset

of necrotic enteritis in drug-free reared broiler chickens. The impact of water application of hop acids from day of hatch is not known. Cornelison *et al*.[7] administered whole hops (0.6% α-acids and 9.3% β-acids) milled into feed over the 42 day experimental duration at rates of 23, 46, 69 and 93 mg/kg. At a rate of 0.5 lb of hops per ton of feed, test birds compared similarly in feed conversion and efficiency to those on feed containing 50 g of penicillin per ton of feed. Both penicillin- and hops-treated groups performed significantly greater than the negative control feed groups in maintaining feed conversion efficiency. No microbial challenge was administered in the aforementioned study and, as whole hop flowers were administered in the diets, activity of specific hop compounds is not discernible. Hanske *et al*.[20] demonstrated that the consumption of the hop-derived antimicrobial xanthohumol does not alter the rat gut microflora. To the best of our knowledge, such a study has not yet been published for hop antimicrobials in the avian intestine.

Previous workers demonstrated enhanced bacterial spore heat inactivation by lupulone addition.[21] Spore formation by type A *C. perfringens* and its role in pathogenicity or growth suppression have not been well studied in chickens. A more complete understanding of the anticlostridial activity of hop components *in situ* should include examination of both the vegetative and spore state of the pathogen.

Short-term administration of therapeutic antimicrobials to broiler chickens is considerably simpler when done through a central water supply vs. formulation into feed. Hence, the current experimentation was conducted using a water route of administration. Direct applications of liquid-based lupulone to dried feed would require an understanding of the biochemical interaction of lupulone with feed components. For example, coating or encapsulation might offer a means of reducing lupulone oxidation and maintain optimal antimicrobial activity within feed and once ingested, within animal digesta. The impact of compound stability, variations in compound formulation, pharmacological effects or impact on the gut microflora are all questions of importance warranting further research into using lupulone as a non-antibiotic antimicrobial alternative for prophylactic or therapeutic applications. In summary, we consider the current body of evidence as impetus to continue the study of lupulone as an antimicrobial for purposes of improved feed conversion on a larger scale or in pathogenicity models of necrotic enteritis.

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Transparency declarations

None to declare.

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